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Thermo-responsive wound dressings by grafting chitosan and poly(*N*-isopropylacrylamide) to plasma-induced graft polymerization modified non-woven fabrics

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ABSTRACT

To obtain a chitosan wound dressings with temperature-responsive characteristics, polypropylene (PP) non-woven fabric (NWF) was modified by direct current pulsed oxygen plasma-induced grafting polymerization of acrylic acid (AAc) to improve hydrophilicity and to introduce carboxylic acid groups. Conjugation of chitosan and poly(*N*-isopropylacrylamide) (PNIPAAm) followed by using water-soluble carbodiimide as a coupling agent to form a novel bigraft PP-*g*-chitosan-*g*-PNIPAAm wound dressing. The amount of chitosan and PNIPAAm grafted to PP-*g*-chitosan-*g*-PNIPAAm were $83.0 \pm 4.6\ \mu g/cm^2$ and $189.5 \pm 8.2\ \mu g/cm^2$, respectively. The surface chemical composition and microstructure of the NWF were studied by electron spectroscopy for chemical analysis (ESCA) and scanning electron microscopy (SEM). The linkages between AAc, chitosan, and PNIPAAm were confirmed with the formation of amide bonds. Physical properties of the NWF were characterized and potentials of these NWFs as wound dressings were evaluated using SD rat as the animal model. NWFs contained PNIPAAm were better than those contained only chitosan in wound healing rates and the wound areas covered by PP-*g*-chitosan-*g*-PNIPAAm wound dressings healed completely in 17 days.

1. Introduction

Wound healing is a complicated sequence of cellular and biochemical events proceeds through a series of different phases [1]. Instant coverage of wound areas by wound dressings is necessary to complete the wound healing process [2]. Non-woven fabrics (NWFs) are widely used as disposable wound dressings. They are excellent dressing materials with the high porosity, large surface area, and easiness to process. A polypropylene (PP) NWF as the base material for wound dressing will provide an open structure for drainage of exudates to reduce the risk of second infection, and serve as a protective film to prevent the wound from infection, fluid loss, and bacterial invasion [3]. However, PP NWF is hydrophobic, which requires surface modification before it can meet specific demands for the sophisticated application in wound dressing. To activate a hydrophobic PP NWF surface, plasma modification is widely recognized as a clean and effective method.

Chitosan, a bio-copolymer comprising glucosamine and *N*-acetylglucosamine and derived from the exoskeletons of insects and shells of crustaceans, is an alkaline deacetylated product of chitin. Chitin and chitosan have been examined and used in a wide variety of biomedical applications, such as drug delivery carriers, surgical sutures, bone healing materials, and especially wound dressings [4]. Chitosan has been well known as being able to accelerate wound healing in human [5]. Chitin and certain derivatives could accelerate tensile strength of wounds by speeding up collagen synthesis by fibroblasts during the early stage of wound healing [6]. Chitosan could achieve hemostasis and promote normal tissue regeneration [7]. Besides, the biodegradable chitosan itself provides bacteriostatic and fungistatic activities [8]. Since chitosan has become one of the most important biomaterials for wound management in recent years, grafting a bioactive chitosan layer to a PP NWF is potentially a good way to develop a wound dressing.

A major drawback in conventional dressing materials is the adherence between the gauze and the damaged tissue. If the separation between the dressing material and the tissue is difficult during dressing change, a secondary damage to the wound will occur to prolong the wound healing time. A wound dressing with an easily stripped off property is therefore highly desirable to help lightening the pain patients suffered during frequent dressing changes. A method for preparing easily stripped off wound dressings has been developed by graft polymerization of *N*-isopropylacrylamide (NIPAAm) onto NWFs [9,10], with the thermo-sensitive property of NIPAAm polymer [11]. Poly(*N*-isopropylacrylamide) (PNIPAAm) exhibits a lower critical solution temperature (LCST) and remarkable

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hydration–dehydration changes in response to relatively small changes in temperature [12]. Below the LCST which is around 32 °C, PNIPAAm chains hydrate to form an expanded structure; above the LCST, PNIPAAm chains dehydrate to form a shrinkage structure. Poly(*N*-isopropylacrylamide) (PNIPAAm) and its related copolymers are widely investigated thermo-responsive hydrogel polymers, which have been applied for drug delivery and regenerative medicine [13–15]. By grafting PNIPAAm onto the surface of the base material of a wound dressing, the polymer becomes hydrophobic by expelling water with the skin temperature being above the LCST. The PNIPAAm connection layer will adhere well with the tissue in this case when the wound is in the moist condition. During the change of the wound dressing where separation of the dressing material from the tissue is required, a low temperature treatment (below the LCST of PNIPAAm) to the dressing material will make the polymer hydrophilic and absorb more water to swell the polymer layer. In such case, the wound dressing could be readily stripped off from the wound site without damaging the newly regenerated skin tissue.

To obtain chitosan-grafted PP NWFs with temperature-responsive and easily stripped off characteristic, PP NWFs were modified by plasma-induced grafting polymerization of acrylic acid (AAc). Chemical grafting of chitosan and PNIPAAm using water-soluble carbodiimide as a coupling agent were followed to fabricate PP-*g*-chitosan-*g*-PNIPAAm wound dressings.

2. Experimental details

2.1. Preparation of PP-g-chitosan

PP NWF (density = 0.014 g/cm^3, thickness = 370 μm, porosity = 84%, supplied by Taiwan Textile Research Institute) was first modified by the DC-pulsed plasma system developed previously in our laboratory [16]. After 75 s plasma treatment, the NWF (1 cm × 1 cm) was immersed in 20 wt% AAc (Fluka) aqueous solution and shaking at 60 °C for 4 h. The amount of AAc grated to the NWF was determined by Toluidine Blue (Sigma) from 5 samples [17]. After coating AAc on the surface, the NWFs were reacted with 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Acros) in 0.1 M MES buffer (pH 6, Sigma) for 4 h to activate the carboxylic groups, followed by reacting with 5 mg/ml chitosan (MW = 150,000, degree of deacetylation = 98%, Fluka) in 0.5 M acetic acid at 4 °C for 24 h. The chitosan-grafted NWF was washed with 50 ml phosphate buffered saline (PBS) for five times and dried overnight in a vacuum oven at room temperature. Chitosan content was determined by reacting 1 cm × 1 cm chitosan-grafted NWF (or 0.1–0.5 mg chitosan) with 10 ml of 50 μg/ml Remazol Brilliant Red 3BS (Everlight Chemical) solution at 80 °C for 30 min and measuring the absorbance of the dye solution at 541 nm from 5 samples [18].

2.2. Preparation of PP-g-chitosan-g-PNIPAAm

PP-*g*-chitosan-*g*-PNIPAAm was synthesized by conjugating the carboxyl end group of PNIPAAm–COOH to the amino group of chitosan [19]. The average molecular weight of PNIPAAm–COOH is 2.1×10^4 g/mol by end-group titration. Ten milligrams of PNIPAAm–COOH were activated with 2 mg EDC and 4 mg of *N*-hydroxysuccinimide (NHS, Pierce) in 10 ml MES buffer (pH 6) before being reacted with PP-*g*-chitosan. The bigraft PP-*g*-chitosan-*g*-PNIPAAm NWF was thoroughly washed with PBS and dried overnight in a vacuum oven at room temperature. The amount of PNIPAAm grafted to NWF was calculated from the weight difference before and after the grafting reaction using a Mettler Toledo XP56 Microbalance from 5 samples.

Table 1
XPS analysis of surface compositions of NWFs.

Samples	Atomic percent (%)			O/C	N/C
	C	O	N		
Original NWF	98.45	1.55	–	0.016	–
Plasma modified NWF	84.00	16.00	–	0.19	–
AA coated NWF	89.59	10.41	–	0.116	–
PP-*g*-chitosan NWF	83.47	14.52	2.01	0.174	0.024
PP-*g*-chitosan-*g*-PNIPAAm NWF	84.96	10.46	4.58	0.123	0.054

Table 2
The water content of NWFs.

Samples	Water content (%)
Original NWF	2.7 ± 0.2
Plasma modified NWF	376 ± 21
AAc coated NWF	465 ± 15
PP-*g*-chitosan NWF (20 °C)	447 ± 20
PP-*g*-chitosan NWF (40 °C)	454 ± 14
PP-*g*-chitosan-*g*-PNIPAAm NWF (20 °C)	634 ± 15
PP-*g*-chitosan-*g*-PNIPAAm NWF (40 °C)	550 ± 24

The data are presented with mean ± S.D. (N = 8).

2.3. Analysis of NWFs

X-ray photoelectron spectroscopy (XPS) was performed with a PHI 1600 ESCA Spectrometer (Physical Electrons) equipped with a spherical capacitor analyzer and a multi-channel detector. Elemental compositions at surface using C_{1s}, O_{1s} and N_{1s} core level spectra were measured and calculated from XPS peak area with correction algorithms for atomic sensitivity [20]. The NWF was sputter coated with gold and analyzed by scanning electron microscopy (SEM, JOEL JSM 5410).

Cytotoxicity of the material was examined by MTT assay of cell viability according to ISO 10993-5 [21]. NWFs and latex (pieces of latex gloves, positive control) were extracted in Dulbecco's modified Eagle's medium (DMEM) culture medium (Invitrogen) at 37 °C for 24 h and the extracts were collected for cell culture. Fresh culture medium was used as the negative control. 3T3 fibroblasts were seeded (1×10^4 cells/well) in a 6-well tissue culture plate and cultured with 3 ml of various extracts for 7 d at 37 °C in a CO_2 incubator. Cell viability was monitored at 1, 2, 3, 5 and 7 d by MTT assays [16]. Measurements were made for 6 samples.

The equilibrium water content of a NWF sample was measured by immersing the dry sample in distilled water for 4 h to reach equilibrium. The equilibrium water content (%) was calculated by $[(W_w - W_d)/W_d] \times 100\%$, where W_d and W_w are the dry weight and equilibrium wet weight of the sample, respectively. Measurements were made for 8 samples.

The antibacterial activities of NWFs were tested against *Staphylococcus aureus* (BCRC 10451) by the bacteria colony counting assays (modified AATCC 100 test method). A 0.8 g NWF sample was placed in a 2 ml liquid culture medium containing a 10 μl concentrated microbe liquid culture. Then, the samples were shaken at 100 rpm for 24 h at 37 °C. A 100 μl solution was taken and diluted to an appropriate dilution factor, and the final diluted solution was

Table 3
The antibacterial efficacy of NWFs.

Sample	*S. aureus* (CFU/ml)	Antibacterial activity (%)
Control	1.88×10^8	–
Original NWF	1.83×10^8	2.73
AAc coated NWF	1.73×10^8	8.01
PP-*g*-chitosan NWF	5.86×10^5	99.7
PP-*g*-chitosan-*g*-PNIPAAm NWF	5.45×10^6	97.1

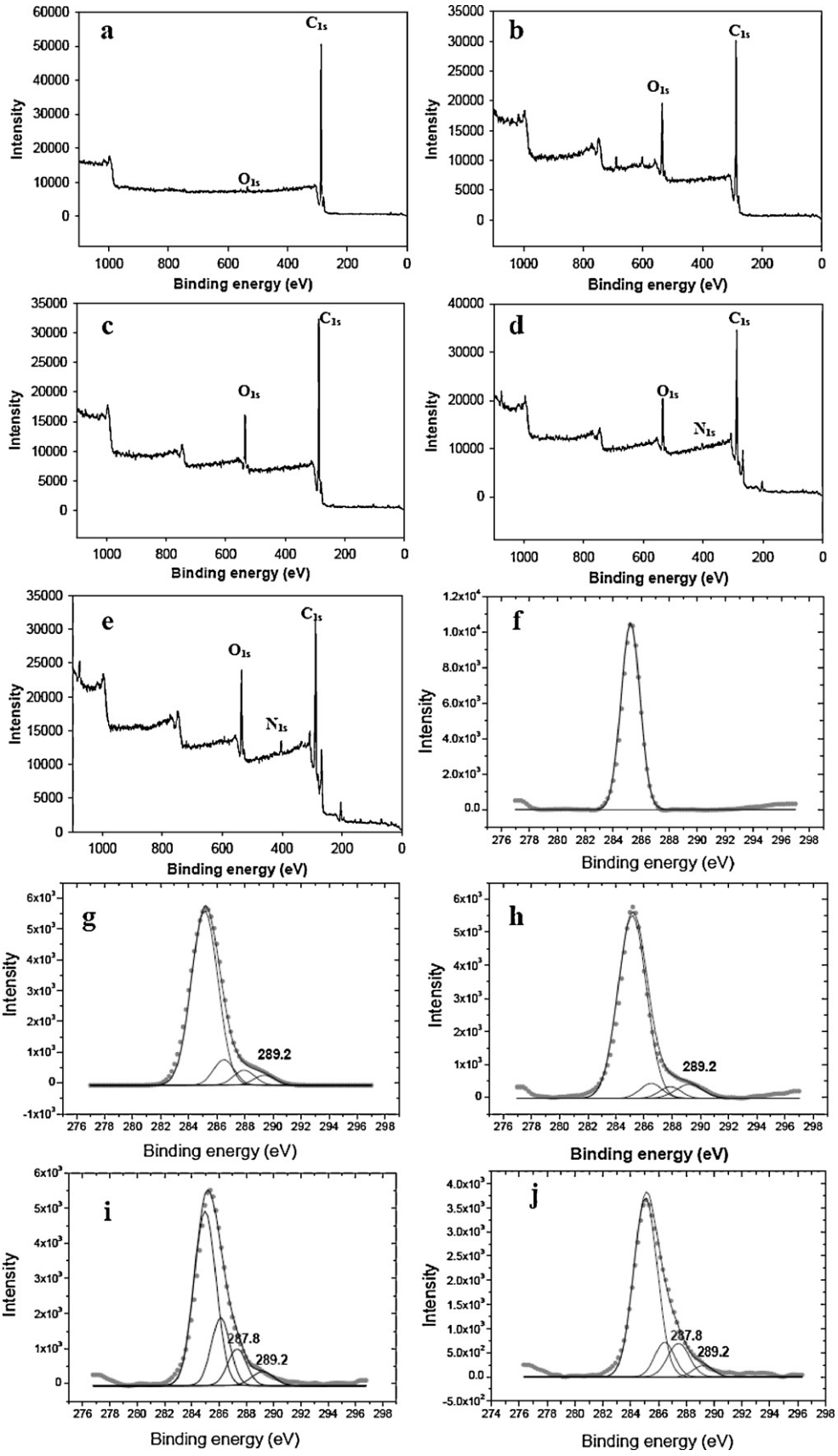

Fig. 1. XPS survey scan spectra (a–e) and high resolution XPS C_{1s} spectra (f–j) of (a and f) original NWF, (b and g) plasma modified NWF, (c and h) AAc coated NWF, (d and i) PP-*g*-chitosan NWF, and (e and j) PP-*g*-chitosan-*g*-PNIPAAm NWF.

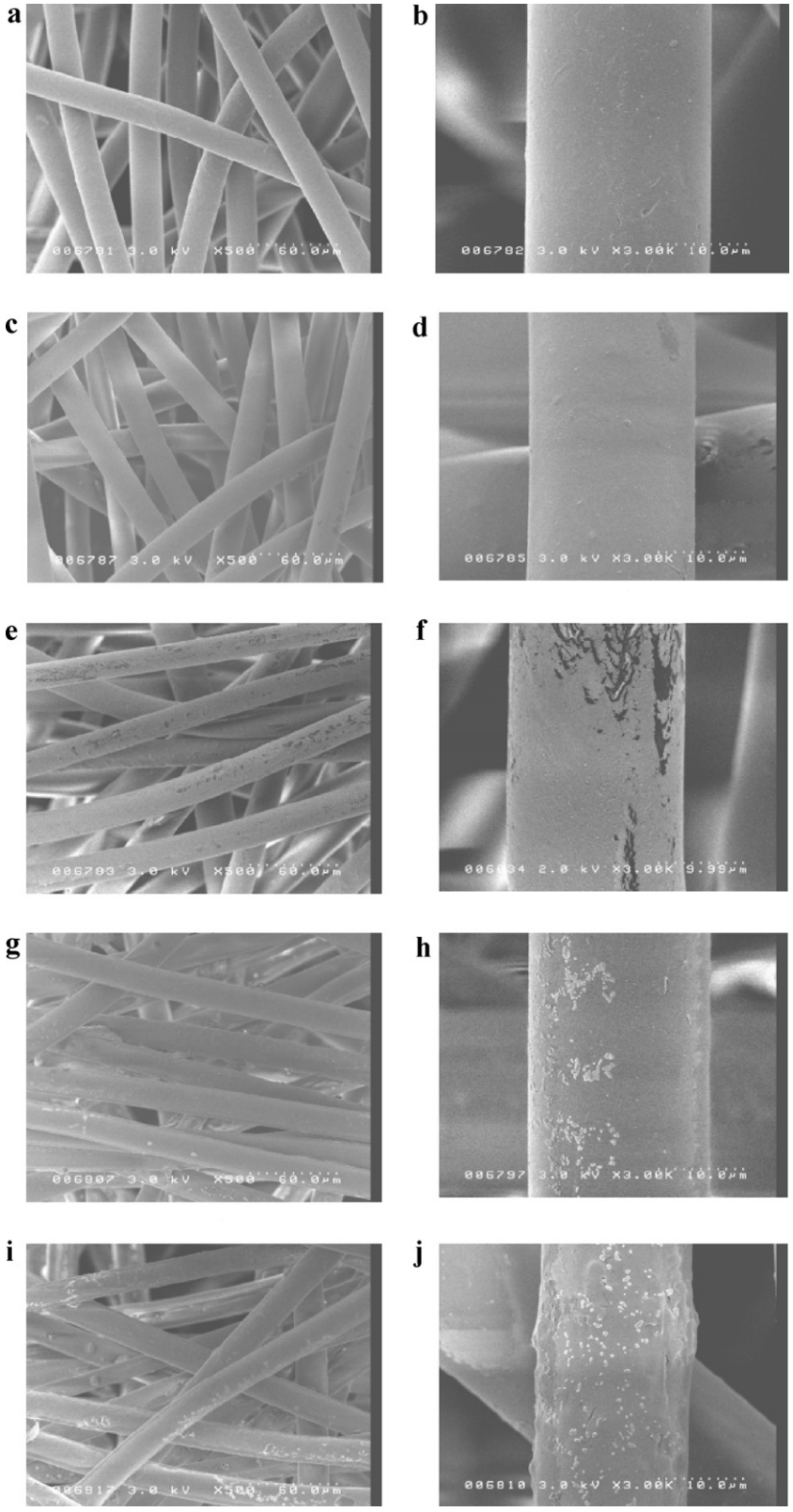

Fig. 2. The scanning electron microscope (SEM) micrographs of (a and b) original NWF, (c and d) plasma modified NWF, (e and f) AAc coated NWF, (g and h) PP-*g*-chitosan NWF, and (i and j) PP-*g*-chitosan-*g*-PNIPAAm NWF.

plated onto a nutrient agar plate. The plate cultured without NWF under the same condition was used as a blank control. All plates were incubated for 24 h at 37 °C and the numbers of the colonies that formed were counted. The antibacterial efficacy of the NWF was calculated according to the following equation, antibacterial activity (%) = $[(A - B)/A] \times 100\%$, where A and B stand for the colony forming unit (CFU) on the plates containing the blank control and the sample, respectively.

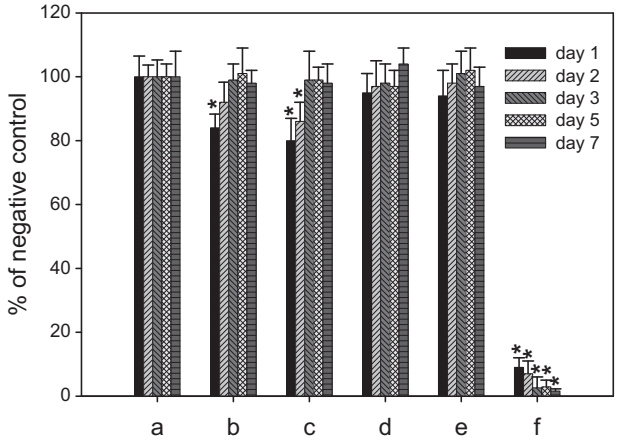

Fig. 3. Cytotoxicity tests from MTT assays of cell viability. (a) Fresh culture medium (negative control), (b) original NWF, (c) AAc coated NWF, (d) PP-*g*-chitosan NWF, (e) PP-*g*-chitosan-*g*-PNIPAAm NWF, and (f) latex (positive control). The absorbance was normalized with that of negative control at each time interval, which is taken as 100%. The data are presented with mean ± S.D. ($N = 6$). *$p < 0.05$ compared with negative control.

2.4. Animal study

Wound healing test was carried out with animal models using SD rats [22]. All experiments were approved by Chang Gung University's Institutional Animal Care and Use Committee and follow the guidelines of experimental animal care. A full thickness wound with a surface area of 2 cm × 2 cm was cut from the back of a SD rat. The wound was covered with an equal size of NWF, gauze (negative control), and commercial wound dressing (Skin Temp II, positive control). The area of the wound was measured at 3, 7, 10, 14, and 17 d after applying cold treatment at 4 °C, which is below the LCST of PNIPAAm, to the wound site for 3 min before removing the wound dressing. The percentage of wound healing is defined as $(D/C) \times 100\%$, where C is the initial wound area and D is the wound area after a fixed time interval. Measurements were made for 4 animals. Skin samples containing the whole wound area were removed after 3 and 17 d post-treatment time, and all specimens were immersed in 10% formalin. They were dehydrated in a graded series of ethanol and were embedded in paraffin. Five to seven micrometer thin sections were prepared and stained with hematoxylin and eosin. Photomicrographs were taken using an Olympus IX-71 microscope.

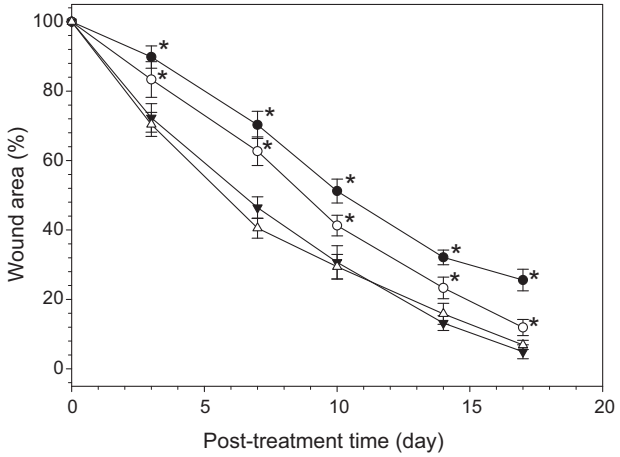

Fig. 4. Wound healing tests of (●) gauze, (○) PP-*g*-chitosan NWF, (△) commercial wound dressing (Skin Temp II), and (▼) PP-*g*-chitosan-*g*-PNIPAAm NWF. The data are presented with mean ± S.D. ($N = 4$). *$p < 0.05$ compared with PP-*g*-chitosan-*g*-PNIPAAm NWF.

3. Results and discussion

The influences of AAc grafting time and grafting concentration on the amount of AAc grafted were first studied. The efficiency of AAc grafting increased with grafting time from 1 to 4 h and reached a plateau at 26.6 ± 0.9 nmol carboxyl groups/cm^2 when reacted with 10% AAc. Using 4 h as the grafting time, the grafted —COOH amount increased with AAc concentration from 5 to 20% but decreased rapidly thereafter due to self-polymerization of AAc above this concentration, which decreased the grafting efficiency. The maximum amount of —COOH that could be introduced to NWF in this case was 43.4 ± 1.2 nmol carboxyl groups/cm^2. This value is high enough for introducing a continuous bioactive chitosan layer on the microfiber surface of the NWF [23]. Chitosan was immobilized to the AAc-coated NWF by using the activation reagent EDC. The amount of chitosan grafted to NWF increased with chitosan concentration until 5 mg/ml and reached a saturation value at 83.0 ± 4.6 μg/cm^2. For further grafting of PNIPAAm–COOH to chitosan-containing PP NWF, a similar carbodiimide-mediated amide bond formation approach was used by adding NHS in addition to EDC. EDC formed an unstable activated acid intermediate with PNIPAAm–COOH and a less labile activated acid could be obtained with the addition of NHS. The amount of PNIPAAm to NWF reached a maximum value at 189.5 ± 8.2 μg/cm^2 if reacted with ≧10 mg/ml PNIPAAm–COOH.

XPS was used to characterize the surfaces and the survey scan spectra and the high resolution C_{1s} peaks are shown in Fig. 1. The significant increase (3.8% to 6.3%) of C_{1s} peak intensities at 289.2 eV (O═C—O) after AAc coating can be related to the increase of carboxyl groups on the surface (Fig. 1g and h). For the chitosan and PNIPAAm grafted NWF, a significant increase of peak intensity at 287.8 eV (increase from 5.2% to 7.8% after chitosan grafting and further to 11.8% after PNIPAAm grafting) can be ascribed to the formation of amide bonds (NH—C═O) between AAc and chitosan, chitosan and PNIPAAm–COOH, and the amide bonds in PNIPAAm (Fig. 1i and j). The peak intensity for the O═C—O group at 289.2 eV also decreases notably from 6.3% to 3.7% after chitosan grafting owing to the participation of this functional group in AAc in amide bond formation (Fig. 1h and i).

As shown from Table 1, the oxygen to carbon (O/C) ratio substantially increases after plasma modification as a result of introducing highly oxidative oxygen-containing radicals, and this value decreases after introducing AAc to the surface (Fig. 1a–c). The O/C ratios changes again during the following grafting reactions of chitosan and PNIPAAm (Table 1). The element N_{1s} peak appears after grafting chitosan since it is a polysaccharide rich in the amino sugar glucosamine (Fig. 1d). The nitrogen to carbon (N/C) ratio increases after PNIPAAm grafting with the abundance of N in the polymer (Fig. 1e).

The surface morphology of the NWFs was studied using SEM (Fig. 2). The microfiber in PP NWF after plasma treatment (Fig. 2c and d) remains around 20 μm in diameter and appears to have a smooth surface morphology as for original PP NWF (Fig. 2a and b). A thin layer of polymer was evident on the surface of NWF microfiber after AAc coating (Fig. 2e and f). The surface morphology of NWF changed substantially after chitosan (Fig. 2g and h) and PNIPAAm (Fig. 2i and j) grafting, with concomitant increase in surface roughness after each grafting step.

The water absorbance capacity of the NWF enhances drastically after plasma modification and AAc modification due to the change in surface hydrophilicity (Table 2). This value further increases after subsequent grafting of chitosan and PNIPAAm

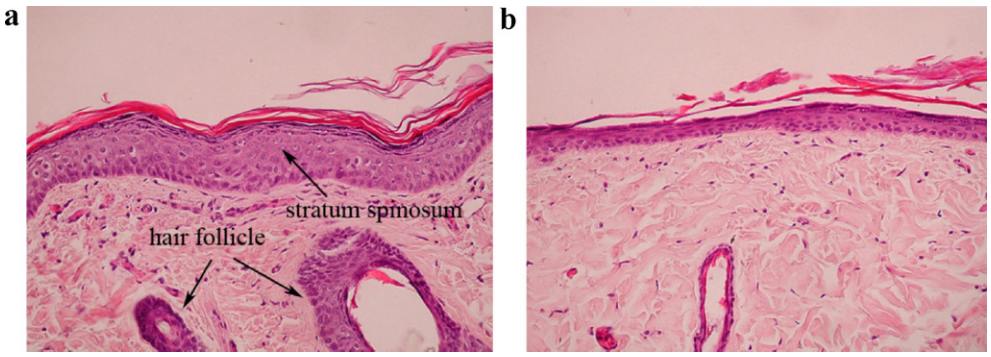

Fig. 5. Histological photomicrographs of the newborn skin tissue at the wound site after covered with PP-*g*-chitosan-*g*-PNIPAAm NWF for (a) 3 days and (b) 17 days.

(Table 2). This property can make PP-*g*-chitosan-*g*-PNIPAAm NWF a good absorptive material for wound drainage at the wound site and further enhances its potential feasibility as wound dressing. It should be noted that the water content of the NWF is temperature-dependent after PNIPAAm grafting, which is not observed for PP-*g*-chitosan NWF (Table 2). This behavior is a result of the temperature-responsive nature of grafted PNIPAAm polymer chains, which demonstrates the successful introduction of the thermo-sensitive characteristic to the NWF wound dressing. The LCSTs for PNIPAAm–COOH used in this study is 29.8 °C while that of chitosan-*g*-PNIPAAm is 29.3–30.3 °C, depending on the grafting ratio [19]. As the skin temperature of human skin is above 32 °C, the PNIPAAm polymer chain grafted to the wound dressing is expected to be in a collapsed state during wound dressing application and in a expanded state during wound dressing removal, which can meet the need for an easily stripped off wound dressing.

Results of *in vitro* cytotoxicity tests are given in Fig. 3. Some cytotoxicity was observed for the original and AAc coated NWFs, where absorbance was less than that of the negative control (fresh culture medium) during the initial stage of cell culture. However, by grafting with chitosan and PNIPAAm, the cytotoxicity has been reduced. Temperature-responsive PP-*g*-chitosan-*g*-PNIPAAm NWF therefore shows good *in vitro* biocompatibility and support proliferation and normal functions of fibroblasts.

The antibacterial activity of the NWF against *S. aureus* was determined by the plate counting method. As shown from Table 3, for original and AAc coated NWFs, the antibacterial activity is negligible. Excellent antibacterial properties were found for PP-*g*-chitosan and PP-*g*-chitosan-*g*-PNIPAAm NWFs where the antibacterial activity increased to 99.7% after chitosan grafting and decreased slightly to 97.1% after further PNIPAAm grafting. These results certainly reflect that the antibacterial properties of modified NWFs are mainly attributed to the existence of chitosan. Chitosan is well known for its antimicrobial activity against a variety of bacteria and fungi, which is originating from its polycationic nature at physiological pH (NH_3^+ group) [24]. The interaction between positively charged chitosan and negatively charged microbial cell wall leads to the leakage of intracellular constituents. The binding of chitosan with DNA and inhibition of mRNA synthesis also occur with the penetration of chitosan into the nuclei of the microorganisms to interfere with the synthesis of mRNA and proteins. Since PNIPAAm grafting requires the participation of —NH_2 group of chitosan, the positively charged NH_3^+ will decrease for PP-*g*-chitosan-*g*-PNIPAAm NWF hence influence its antibacterial activity.

Fig. 4 shows the changes in the wound area at different post-treatment time. The wound areas decreased gradually and reached 11.9% and 4.9% after 17 d for PP-*g*-chitosan and PP-*g*-chitosan-*g*-PNIPAAm NWFs, respectively. In contrast, for wound covered with gauze, the wound area only reduced to 25.6% during the same period. Also, the wound closure rate of PP-*g*-chitosan-*g*-PNIPAAm shows no statistical difference from that of a commercial wound dressing. Introducing temperature-responsiveness to PP-*g*-chitosan could therefore augment the wound healing capability of chitosan-grafted wound dressing. The histological observation of the skin samples after covered with PP-*g*-chitosan-*g*-PNIPAAm for different times also confirms that no infection and inflammation occurs at the wound site throughout the wound healing process (Fig. 5).

4. Conclusions

A wound dressing was developed in this study by sequential grafting PNIPAAm and chitosan to an AAc coated NWF after plasma modification. With its biocompatibility, anti-bacterial activity, temperature-responsiveness, and wound healing ability, this NWF will be useful as a potential wound dressing material for biomedical use.

Acknowledgments

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